Medical Science

The role of Dihydroartemisinin in suppression of experimental Dexamethazone-Induced Osteoporosis in adult female albino rat

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ABSTRACT

Background: Osteoporosis is a major public health problem leading to morbidity and mortality in many individuals. Osteoporosis is one of the main complications of glucocorticoid (GC) application. Currently, the employ of medical herbs has emerged as one of the main popular and preferred complementary and traditional therapy. Evidence provided that certain vegetables and fruits are essential for maintaining bone mass and preventing osteoporosis. Artemisia annua is a Chinese traditional herb which was used safely for long time in treatment of malaria. Artemisinins possess anti-inflammatory, anti-oxidant, anti-adipogenic, anti-cancer and anti-microbial activities. Dihydroartemisinin (DHA) is the active metabolite of all artemisinin compounds. *Aim of the study*: This study was designed to link these documented prophits of dihydroartemisinin to the management of dexamethazone induced osteoporosis. *Materials & methods*: This study was designed to link these documented prophits of dihydroartemisinin to the management of dexamethazone induced osteoporosis. *Materials & methods*: 18 female adult albino rats aged 3 months were divided into 3 groups; control, Dexamethazone and Dexamethazone + Dihydroartemisinin 20 mg. We evaluated the osteoporotic changes by histopathology (HE staining, masson-goldner and immunological examination of Beta catenin (Bone formation marker), RANKL (Bone resorption marker) in lumbar vertebrae. *Conclusion*: Dexamethazone clearly inhibited bone formation and induced osteoclastogenesis as proved by masson-goldner staining and immunological staining. The osteoporotic changes were reversed with dihydroartemisinin treatment. We have demonstrated that treatment with dihydroartemisinin at the same time with dexamethazone has significantly improved the histological bone indices as compared to dexamethazone osteoporotic group. Dihydroartemisinin may be a new treatment strategy for the prevention of glucocorticoids-induced osteoporosis.

Keywords: osteoporosis, Dexamethazone, Dihydroartemisinin, albino rats.

1. INTRODUCTION

Dexamethasone (Dexa) 1 is a type of glucocorticoids (GCs) extensively used as a treatment of allergic disorders, ulcerative colitis, arthritis, pulmonary disorders and organ transplantation, owing to its potent anti-inflammatory and immunomodulatory effects (Vandewalle et al., 2018). In spite of the therapeutic effectiveness of this drug, its frequent use inevitably produces variable health problems. Among which, is a severe form of secondary osteoporosis affecting 30-50% of patients with GCs therapy (Nuti et al., 2019). The pathological mechanisms of GCs-induced osteoporosis include diminished bone formation by decreasing differentiation and maturation of osteoblasts and increasing life span of osteoclasts (Li et al., 2015). Osteocytesare also influenced, with reduced cell function and enhanced apoptosis leading to weakness of their capacity to reveal and repair bone microdamage (Fraser & Adachi, 2009). The combination of increased bone resorption and attenuated bone formation may thus explain early and fast loss of bone mineral density (BMD) and bone strength/quality inpatients undergoing GCs therapy (Dobrowolski et al., 2017).

Currently, the employ of medical herbs has appeared as one of the main popular and best ways in complementary and traditional therapy. Evidence provided that certain vegetables and fruits are essential for maintaining bone mass and preventing osteoporosis (Rajput et al., 2018). Artemisinin is well established for the treatment of many form of malaria. It belongs to the family of sesquiterpene lactones, which are derived from extracts of sweet wormwood (Artemisiaannua). Dihydroartemisinin (DHA) is a water-soluble semi-synthetic derivative of artemisinin and it is sold commercially in combination with piperaquine as functional treatment for malaria with little side effects (Zhou et al., 2016).

DHA has also been established to have restrained impacts on tumor cells, particular by modifying the NF-kB pathway (Lee et al., 2012). Receptor for activation of nuclear factor Kappa B ligand (RANKL) is recently known as famous purpose for the curing of bone weakness, due to the presentation of RANKL employing antibodies, peptides, and natural compounds could inhibit osteoclast formation and function (Hwang et al., 2010; Zhou et al., 2016). The current investigation was carried out to define the impact of natural compounds on preventing RANKL-stimulated osteoclast formation and function. Osteoblasts are the main active cells included in bone developing through bone metabolic activities. The Wnt signal transduction pathway is stimulated in the nucleus during β-catenin and is substantial in the osteoblast differentiation and proliferation procedures.

Among the included proteins, Wnt3a as starting factor of the imitative Wnt/β-catenin pathway is vital to osteoblast proliferation and differentiation (Fan et al., 2016). Wnt-β-catenin is an important pathway of osteoblastogenesis. β-catenin is a main aspect of the Wnt signaling pathway. It generally exists in the cytoplasm, but also in the cell membrane and nucleus. In the cytoplasm, while the Wnt signaling pathway is passive, β-catenin is phosphorylated, which in turn activates the ubiquitin system, resulting in its deterioration during the proteasomal pathway (Fan et al., 2016). In previous research DHA showed positive effect on inhibition of osteoclastogenesis (Feng et al., 2016; Ge et al., 2018). In the current trial, we examined the function of DHA in osteoporosis in vivo employing glucocorticoid induced rat model and to justify the role of dihydroartemisinin as a novel therapy in osteoporosis management.

2. MATERIALS AND METHODS

Experimental animals

Eighteen adult female Wister albino rats with average age three months weighting 200-250 gm. were used in this study. This work was carried out at Mansoura Experimental Research Center (MERC) according to rules and regulation 2 laid down by the committee on animals' experimentation of Mansoura Faculty of Medicine, Egypt. Rats were preserved under controlled circumstances of temperature (23 ± 3 ºC), and relative humidity during adaptation and experimental time, and fixed 12:12-hours light/dark cycle. Rats were allowed free reach to nutrients and water. The experiment was carried out from 1 July 2020 to 30th September 2020.

Experimental design and treatments

Rats were randomly split into three groups (6 rats in each group):

Control group (n = 6): received intraperitoneal injection of 0.2ml saline every 2 days for 3 months. Cortisone treated (dexa) group (n=6): received intramuscular injection of dexamethasone at 1 mg/kg b. wt. every 3 days (Liu et al., 2011) for 3 months. Dihydroartemisinin treated (D+D20) groups (n = 6): received intramuscular injection of dexamethasone at 1mg/kg b. wt. every 3 days for 3 months in addition to intraperitoneal injection of dihydroartemisinin 20mg/Kg (Ge et al., 2018) dissolved in 0.2ml saline every 2 days for 3 months.

Specimen's collection

At the assigned time, rats were mightily anaesthetized employing intraperitoneal ketamine (90 mg/kg) and xylazine (15 mg/kg) tobe victimized. The skin over the back was incised and the lumbar vertebral columns were exposed, dissected out and cleaned of excess muscles and soft tissues. The caudal lumbar vertebrae (L5 &L6) were detected by their long transverse processes and were related laterally to lumbar plexus (Anderson, 1977). Caudal lumbar vertebrae were fixed in 10% buffered formal saline for 4 days, decalcified by ethyline-diamline tetra-acetic acid (EDTA) (Dumitrescu et al., 2004; Ge et al., 2018) for about 14 days, double embedded in paraffin and paraffin blocks were prepared.

Histological and immunohistochemical stains

Thickened longitudinal sections (about 5μm) of lumbar vertebrae were stained with hematoxylin and eosin (HE) to detect the bone architecture, Masson Goldner for estimation of new bone formation, and immunohistochemical stains (Beta catenin bone formation marker and RANKL bone resorption marker). For immunohistochemical staining; sections on positively charged glass slides were removed from paraffin wax and rehydrated. The sections were washed with distilled water, immersed in 0.1% hydrogen peroxide andthen rinsed 3 times in phosphate-buffered saline (PBS). Protein block was used (five minutes) to block nonspecific background. The sections were washed 2 with distilled water and then rinsed in PBS 3 times. Sections were brooded with the primary antibody at 4º covernight. The primary antibody was either rabbit polyclonal anti-RANKL (DF7006, Affinity Bioscience, USA, at 1/200 dilution) or rabbit monoclonal anti-Beta catenin antibody (IGX4794R-3, Gene Tex International Corporation, USA at 1/100 dilution). After rinsed three times in PBS for 5 minutes, the sections were brooded for twenty minutes with biotinylated goat antipolyvent. The sections were then incubated with conjugated streptavidin for ten minutes, washed one time more in PBS, and lastly brooded with DAB substrate (diaminobenzidine) for three minutes (Mouse and rabbit HRP/DAB (ABC) detection IHC kit, ab64264, Abcam, UK). Finally sections were counter stained with Mayer's haematoxylin for one minute. Entire sections were investigated employing Olympus®CX41 light microscope and Olympus® SC100 digital camera was used to photograph the stained sections.

Quantitative morphometric assessment

Morphometric examinations were archived employing Image J program (version 1.48, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA), according to the program instructions. Five sections (HE or immunostained) at various levels in lumbar vertebrae were investigated from five animals in each group. Bone trabeculae were measured at their midpoint away from their branching areas in HE stained sections. The mean trabecular thickness of five non overlapping fields (at magnification x100) in each section was calculated. The mean area percentage of immune positive reaction was measured in five non overlapping fields (at magnificationx400; area: 0.071 mm2) in each section of RANKL and Beta catenin in bone marrow of lumbar vertebrae as described by Shaalan et al., (2020).

Statistical analysis

The computer program SPSS (Statistical Package for Social Science) version 22 (IBM, USA) was employed to analyze results. Results were summarized in the form of mean ± standard deviation. For comparison between various groups, one way ANOVA test followed by Tukey's post-hoc test was employed. P value < 0.05 was considered as statistically significant. All graphic representations of the results were archived by Microsoft Excel for windows (Microsoft Inc., USA).

3. RESULTS

Effect of dihydroartemisinin on lumbar vertebrae structure

HE stained sections from lumbar vertebrae cancellous bone of control group showed a lattice of bony trabeculae detached by interconnecting distances including bone marrow formed of hematopoietic tissue and scattered adipocytes (Fig. 1 A1, A2). Dexa group trabecular bone showed obvious rise in the bone marrow adipocytes in comparison to control group. Marked trabecular bone resorption appeared in dexa group indicated by widely separated thin bone spicules. Osteoclasts with acidophilic cytoplasm were housed in the eroded resorption area in trabecular bone (Fig. 1 B1, B2). The effect of dexamethasone reversed in treated group D+D20 indicated by increase in thickness of trabeculae, decrease numbers of adipocytes and osteoclasts compared with dexa group (Fig .1 C1,C2). Statically, trabecular bone thickness in dexa group (51.86±7.07) was high significantly (P<0.001) decreased compared to that of control group (161.55 ± 8.12). Trabecular bone thickness in treated groups (D+D20, 125.04± 16.29) was high significantly

(P< 0.001) increased compared to that of dexa group. In comparison to control, D+D20 treated group showed high significant (P< 0.001) decrease (Fig.1D).
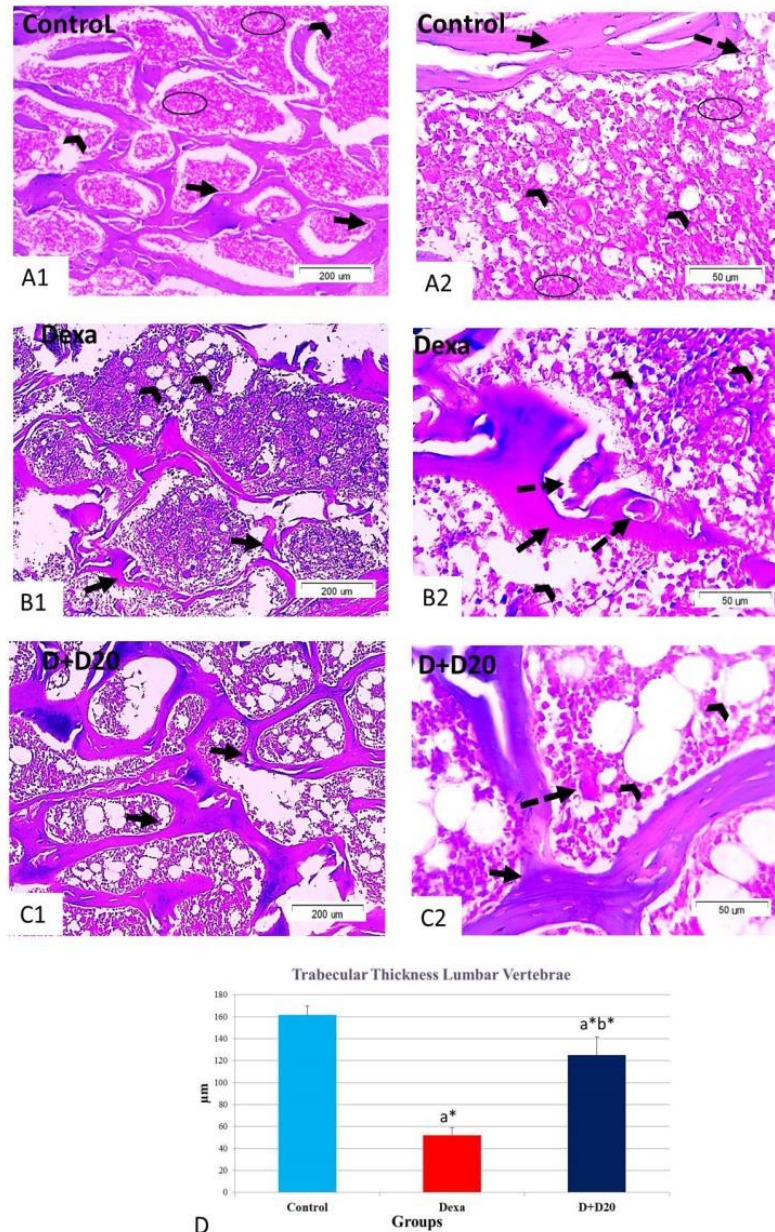

Figure 1 Photomicrographs of trabecular bone of lumbar vertebrae sections stained with HE: (A1, A2) Control group shows porous bone composed of a lattice of bony trabeculae (black arrows) detached by interconnecting distances including bone marrow formed of hemopoietic tissue (circles) and scattered adipocytes (arrow heads) (B1, B2) Dexa group shows obvious enhance in the bone marrow adipocytes (arrow heads) in comparison with control group. Marked trabecular bone resorption appears in dexa group indicated by widely separated thin bone spicules (black arrow) and osteoclasts inside resorption cavities (dashed arrows) (C1, C2) The effect of dexamethasone reversed in treated group D+D20 with obvious increase in thickness of trabeculae (black arrows), decrease numbers of adipocytes (arrow heads) and osteoclasts (dashed arrows) as compared with dexa group (Original magnification A1, B1, C1 x 100; Scale bar= 200 μm and A2, B2, C2 x400; Scale bar= 50 μm) (D) Histogram illustrating the trabecular thickness in the different groups. a significant (P < 0.05) versus the control group; b significant (P < 0.05) versus dexa group; * high significant (P < 0.001).

Effect of dihydroartemisinin on bone turnover of lumbar vertebrae

Masson Goldner stained sections of lumbar vertebrae compact and trabecular bone showed normal appearance of green stained mature bone and normal amount of red stained newly formed bone were observed in control group (Fig. 2 A1, A2). Marked reduction of red stained newly formed bone appeared in Dexa group (Fig. 2 B1, B2). The effect of dexamethasone reversed in treated group D+D20 with obvious increase of red stained newly formed bone when compared with the dexa group (Fig.2 C1, C2).

Effect of dihydroartemisinin on RANKL immune-reactivity in bone marrow of lumbar vertebrae

RANKL expression was nearly absent from bone marrow in control group (Fig. 3A). Strong expression against RANKL was seen in bone marrow from Dexa group (24.66±3.32) with highly significant (P< 0.001) increase in area percentage compared to that of control group (0.00±0.00) (Fig. 3B, D). RANKL expression in bone marrow decreased in treated groups (D+D20, 4.16± 3.76) with highly significant (P<0.001) decrease in area percentage when compared with the dexa group. The area percentage of RANKL expression became non-significantly different from control group in D+D20 treated group (Fig. 3C, D).

Effect of dihydroartemisinin on Beta catenin immuno-reactivity in bone marrow of lumbar vertebrae

B-catenin expression was detected in bone marrow in control group (fig. 4A). B-catenin expression was absent in bone marrow from Dexa group (0.00±0.00) with highly significant (P< 0.001) decrease in area percentage compared to that of control group (35.67 ± 6.32) (Fig. 4B, D). B-catenin expression increased in the bone marrow of treated group D+D20 when compared with the dexagroup with highly significant (11.50± 10.07) (P<0.001)increase in area percentage when compared with the dexa group (Fig. 4 C, D).
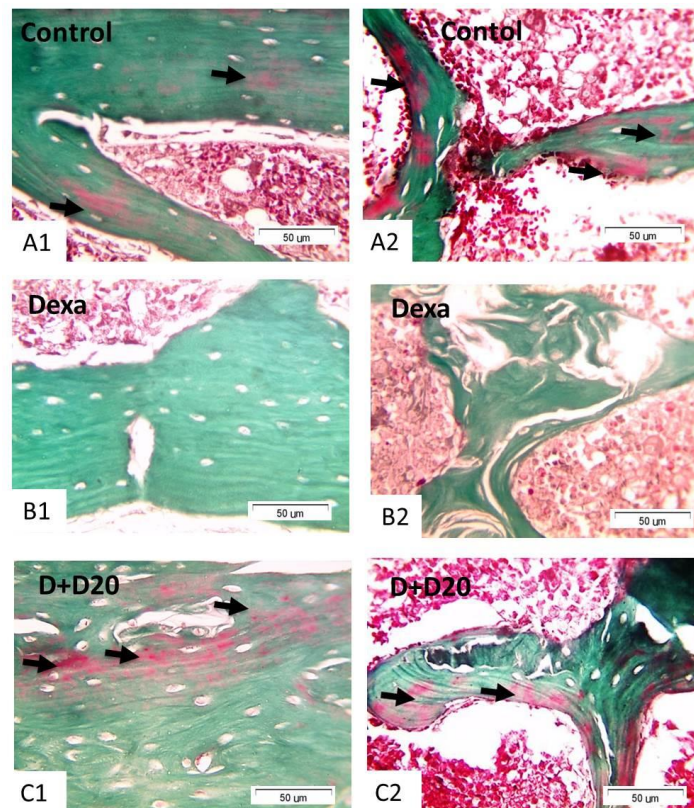

Figure 2 Photomicrograph of lumbar vertebrae compact bone (A1, B1,C1) and cancellous bone (A2,B2,C2) sections stained with Masson Goldner: (A) Control group shows normal appearanceof green stained mature bone and normal amount of red stained newly formed bone (black arrows) (B) Dexa group demonstrates marked reduction of red stained newly formed bone(C) The effect of dexamethasone reversed in treated group D+D20 with increased red stained newly formed bone (black arrows) when compared with the dexa group (Original magnification x400, bar 50).
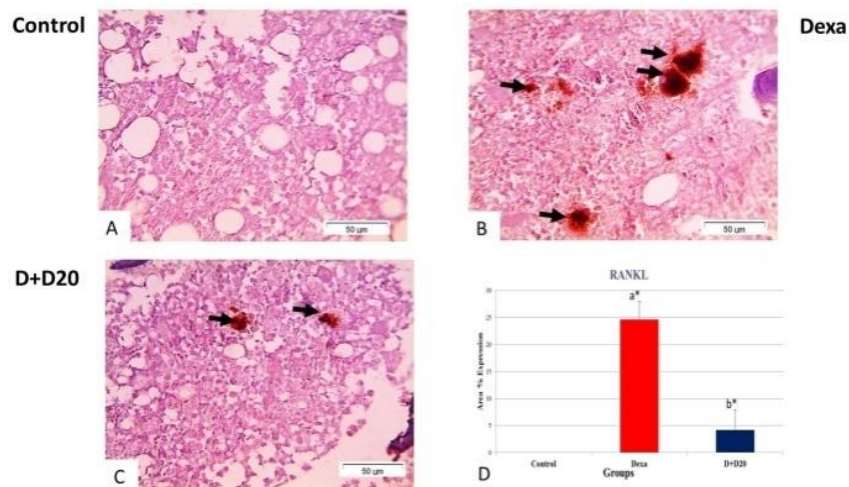
Figure 3 Photomicrographs of RANKL-immunostained bone marrow of lumbar vertebrae sections: (A) In control group RANKL expression is absent (B) Dexa group shows strong expression against RANKL (C) RANKL expression in bone marrow decreased in treated group D+D20 when compared with the dexa group (black arrows point to positive reaction in bone marrow). (Original magnification x400; Scale bar= 50 μm)(D) Histogram clarifying the area percentage expression of RANKL in the different groups. a significant (P < 0.05) versus the control group; b significant (P < 0.05) against dexa group; *high significant (P < 0.001).
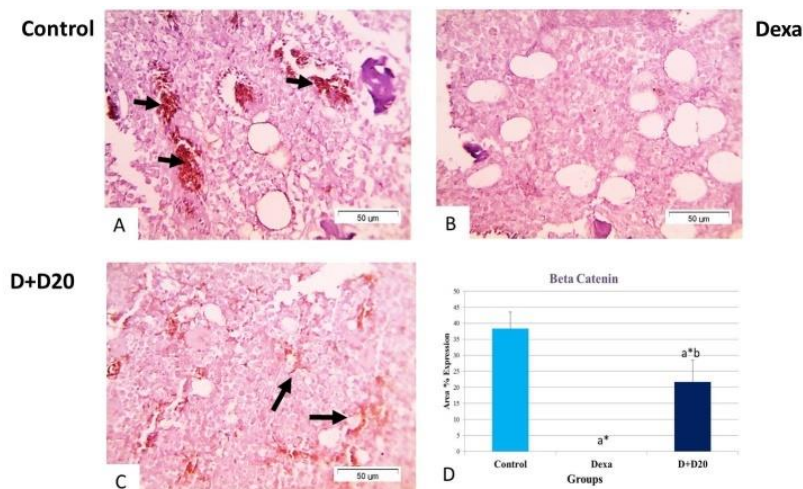

Figure 4 Photomicrographs of B-catenin immunostained bonemarrow of lumbar vertebrae sections: (A) In control group B-catenin expression is detected in bone marrow (B) B-catenin expression is absent in Dexa group bone marrow (C) B-catenin expression increased in bone marrow of treated group D+D20 when compared with the dexa group (black arrows point to positive reaction in bone marrow). (Original magnification x400; Scale bar=50 μm) (D) Histogram clarifying the area percentage expression of beta catenin in the different groups. a significant (P < 0.05) versus the control group; b significant (P < 0.05) versus dexa group; * high significant(P < 0.001)

4. DISCUSSION

Bisphosphonates are the first line therapy for osteoporosis till now. Among bisphosphonates, alendronate is the most commonly used drug for prevention and treatment of different types of osteoporosis (Kim et al., 2016). Despite the positive effects of alendronate on bone histology and BMD, the increased MDA level of oxidative stress during treatment can cause kidney damage and gastrointestinal adverse effects during its longer administration (Oršolić et al., 2018). Artemisinin possess anti-inflammatory, anti-oxidant, anti-adipogenic, anti-cancer and anti-microbial activities. DHA is the active metabolite of all artemisinin derivatives (Ferreira et al., 2010). The present study tried to link this documented anti-resorptive and anti-oxidant effects of Dihydroartemisinin to the management of glucocorticoid- induced osteoporosis in rats. Many medical disciplines require treatment with GCs with no

alternatives due to their anti-inflammatory and immunosuppressive effect. However, osteoporosis and related fracture are serious complications with long term GC therapy. They inhibit calcium transport and cause secondary hyperparathyroidism, hypogonadism and impairment of osteoblast function. In addition, using GCs in pre-menopausal women makes them at significant risk of developing glucocorticoids-induced osteoporosis (Hsu & Nanes, 2017). Individuals cured with GCs have been described to have an early, fast rise in bone resorption accompanied by a prolonged depletion in bone formation (Canalis et al., 2004).

In the present study, dexamethasone treatment led to micro-architectural changes of bone indicating osteoporotic-like changes. As regards spongy bone, it revealed widening of the bone marrow space with few bone trabeculae which appeared thinned out and were seen as islands of widely separated spicules and increased osteoclastic activity and consequently, increase in bone resorption. Our results are in agreement with El-Morsy et al., (2011) & Bouvard et al., (2013). The current data indicates that HE may have ameliorates impacts versus glucocorticoid-stimulated bone resorption. The employed of DHA in the medication of osteoporosis cleared improvement of lumbar vertebrae spongy bone histology. Spongy bone of lumbar vertebrae revealed increased thickness of the bony trabeculae with less apparent widening in their bone marrow spaces compared to the osteoporotic group. The ability of DHA to induce bone repair was proved by masson-goldner staining which detected new bone formation as red stained areas. Osteoporotic like changes were confirmed statistically in the form of a high significant decrease (P< 0.001) in the trabecularbone thickness of lumbar vertebrae as compared with those of normal control rats. Similar results were reported by Ren et al., (2017) as bone resorption manifestation ofosteoporosis.

In our results, DHA treatment significantly restored trabecular bone thickness of lumbar vertebrae as compared with the osteoporotic group. Lee et al., (2017) who used treated ovariectomized mice model of osteoporosis with artemisinin observed similar results. Glucocorticoid is recognized to stimulate bone weakness mostly through repressing the bone formation-arbitrated osteogenesis. Both the bone morphogenetic protein (BMP)-Runx2 and the Wnt signal pathways are renowned to perform critical functions in bone formation and to induce osteoblast development (Hayashi et al., 2009). β- catenin is a main aspect of the Wnt signaling pathway. It generally exists in the cytoplasm, but also in the cell membrane and nucleus. In the cytoplasm, when the Wnt signaling pathway is passive, β-catenin is phosphorylated, which in turn activates the ubiquitin system, resulting in its deterioration during the proteasomal pathway (Kestler & Kuhl, 2008; Fan et al., 2016).

In our results, statistical analysis of the area % of B-catenin positive expression in bone marrow of lumbar vertebrae revealed high significant decreased expression in dexa groupas compared with that of the normal control group which may indicate inhibition of osteogenesis. This is in agreement with Sousa et al., (2017). The treated with DHA showed high significant increase in area percentage of β-catenin expression as compared with the osteoporotic group. So, our results indicate that DHA can stimulate Wnt-Beta catenin pathway of osteblastogenesis and has the ability of bone repair and new bone formation which was also proved by masson-goldner staining. On the contrary, Feng et al., (2016) reported that DHA does not impact osteoblast development and osteoblast correlated gene expression. Many previous researches explained mechanisms by which glucocorticoid induced osteoporosis. One of them is that GCs enhance the expression of cytokines, involving receptor of activator of NF-kappa b ligand (RANKL), that are including in differentiation of osteoclasts and conversely reduce those included in suppression of osteoclasts, with the net influence of raised bone resorption (Whittier and Saag, 2016). All histomorphometric tests of glucocorticoid cured patients provided the depression of bone mass (Soelaiman et al., 2017).

Allam et al., (2010) recorded that the enhancement in RANKL immune-reactivity is one of the considerable index of osteoclastic activity in bone tissue. In our results, statistical analysis of the RANKL positive expression area % in bone marrow of lumbar vertebrae revealed high significant decrease in dexa group. The expression was significantly increased in the treated group D+D20. This result is in agreement with Zhang et al., (2014). A possible mechanism by which artemisinin decreased RANKL expression may be that artemisinin produces a significant inhibition of the NF-κB canonical pathway activation. This pathway is responsible for TNF-α, IL-1b and IL-6 production in the macrophages (Wang et al., 2011). Activated T cells can express RANKL and various othercytokines that promote osteoclastogenesis (Sato et al., 2006). Artemisinins, also; suppresses the proliferation of T-cell and reduces T-cell-related immune response by decreasing therelease of the IL-2 and TNF-α (Huang et al., 2014). DHA suppress osteoclasts through modifying AKT/SRC signaling pathway. Moreover, DHA has inhibitory effect on NFATc1 which is one significant downstream purpose of RANKL-stimulate bone formation (Feng et al., 2016).

5. CONCLUSION

It could be concluded that Dihydroartemisinin improved osteoporotic-like changes induced by dexa through inhibition of the RANKL-induced osteoclastogenesis and increase of β-catenin expression which might induce osteoblstogenesis and increased new bone formation. Dihydroartemisinin may be a new treatment strategy for the prevention of glucocorticoids-induced osteoporosis

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